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Molecular Medicine

PHAGE LIBRARIES — A NEW ROUTE TO CLINICALLY USEFUL ANTIBODIES

CARA MARKS, PH.D., AND JAMES D. MARKS, M.D., PH.D.

ANTIBODIES can bind with high affinity and specificity to molecules of virtually any shape and to antigens ranging from small organic compounds to large proteins. These characteristics have led to the widespread use of antibodies as laboratory reagents, in diagnostic tests, and for therapeutic purposes. The immune system produces antibodies by a process of natural selection. In this article we describe how the strategy of natural selection can be used in bacteria to produce antibodies with novel characteristics and binding properties. This technology is opening up new diagnostic and therapeutic applications of antibodies.

ANTIBODY PRODUCTION IN VIVO

In vivo, antigen-driven selection governs the production of antibodies (Fig. 1). The process has three key features: the generation of millions of different antibody genes through the rearrangement of a limited number of gene segments; the expression of this repertoire of rearranged genes on the surface of B lymphocytes, where the antibody functions as an antigen receptor; and the antigen-driven selection of clones of B lymphocytes for proliferation and differentiation into antibody-producing plasma cells. High-affinity antibodies are generated by somatic mutation of the antibody genes and further rounds of selection by antigens.

ANTIBODY PRODUCTION IN VITRO

The unique recognition properties of antibodies have been exploited for diagnostic and therapeutic applications since the turn of the century. Initially, polyclonal antibodies from the serum of immunized animals or humans were used. An important advance was the discovery in 1975 that monoclonal antibodies could be produced by hybridomas that were made by fusing a single B lymphocyte, usually obtained from immunized mice, with an immortal cell line. Hybridomas can secrete unlimited quantities of a single antibody with reproducible binding properties.

However, such monoclonal antibodies have limitations, especially with respect to therapeutic applications. Murine monoclonal antibodies are immunogenic in humans, which decreases their efficacy over time and is associated with a risk of allergic reactions. It has been difficult to produce human monoclonal antibodies from hybridomas. Moreover, the murine immune system may fail to recognize medically relevant molecules, particularly those whose structures are similar or identical in mice and humans. There also appears to be a limit to the affinity of murine monoclonal antibodies, which renders them inadequate for many clinical applications.

MIMICKING IMMUNE SELECTION IN VITRO

Three technical advances have made possible antigen-driven immune selection in vitro. First, it has proved possible to express the antigen-binding domains of the heavy (V_H) and light (V_κ) chains of antibodies in *Escherichia coli*, either as Fab fragments or as single-chain Fv (scFv) fragments (Fig. 1). Second, large and diverse repertoires of *Fab* or *scFv* genes can be generated with the polymerase chain reaction (Fig. 2). These repertoires can be built from V_H and V_κ genes obtained from B lymphocytes, either before or after immunization, or from cloned V gene segments that have been rearranged in vitro. Third, the scFv or Fab antibody fragments can be expressed on the surface of viruses (phages) that infect *E. coli*. This step is accomplished by cloning the antibody genes into a phage vector in a way that fuses them with a gene that encodes a protein expressed on the surface of the phage (Fig. 2). The resulting phage anchors the antibody on its surface by means of the coat protein and contains the gene encoding the antibody. The genetically engineered phage thus mimics the B lymphocyte by expressing an antibody on its surface and having an immunoglobulin genotype.

The repertoires of *scFv* or *Fab* genes can be cloned into phage vectors, creating phage-antibody libraries (Fig. 2). Phage antibodies that bind to a particular antigen can be separated from nonbinding phage antibodies by antigen selection. After incubation with immobilized antigen, nonbinding phages are washed away, and the bound phages are recovered by elution from the solid-phase antigen (Fig. 2). A single round of selection increases the number of antigen-binding

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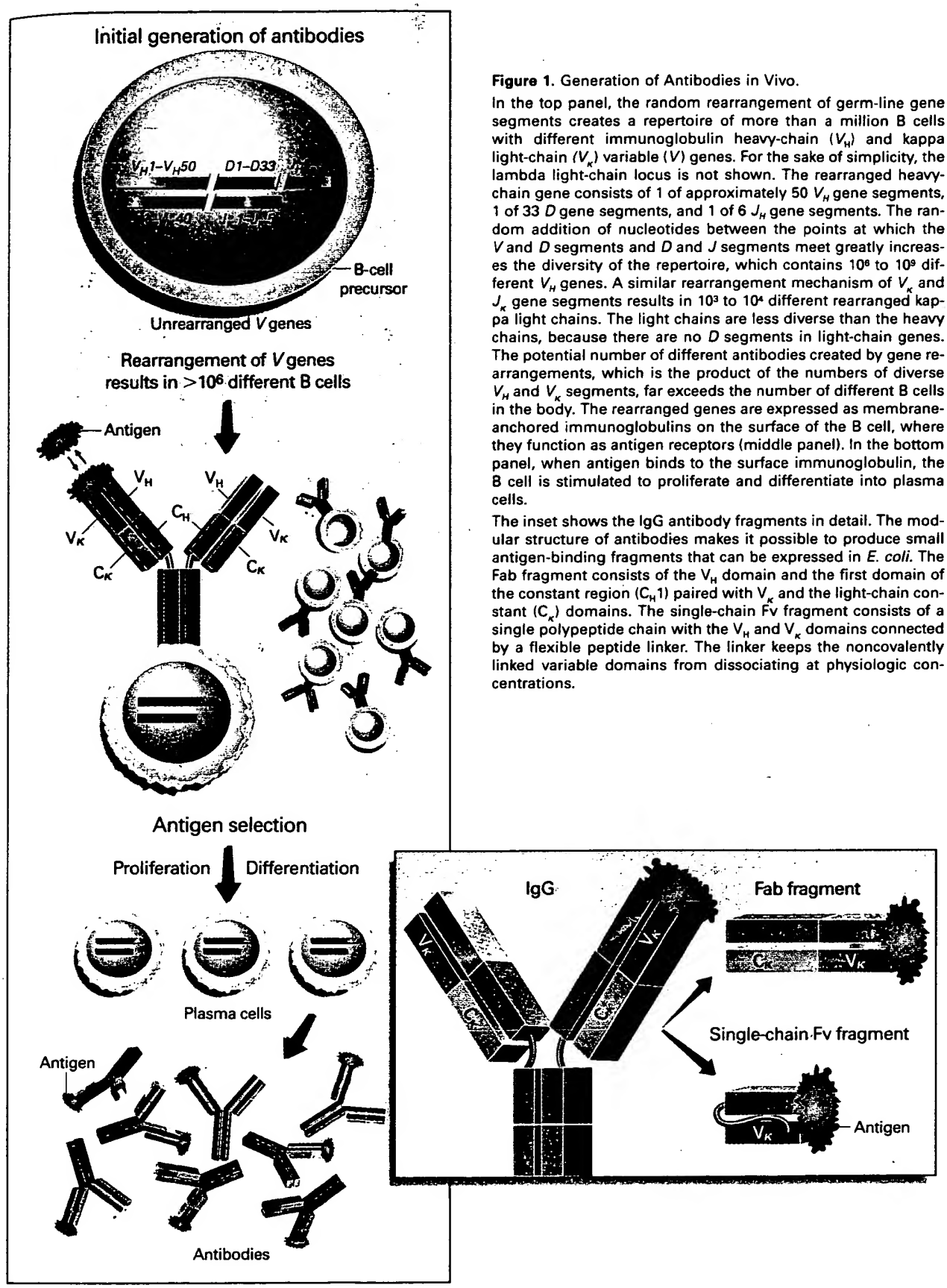
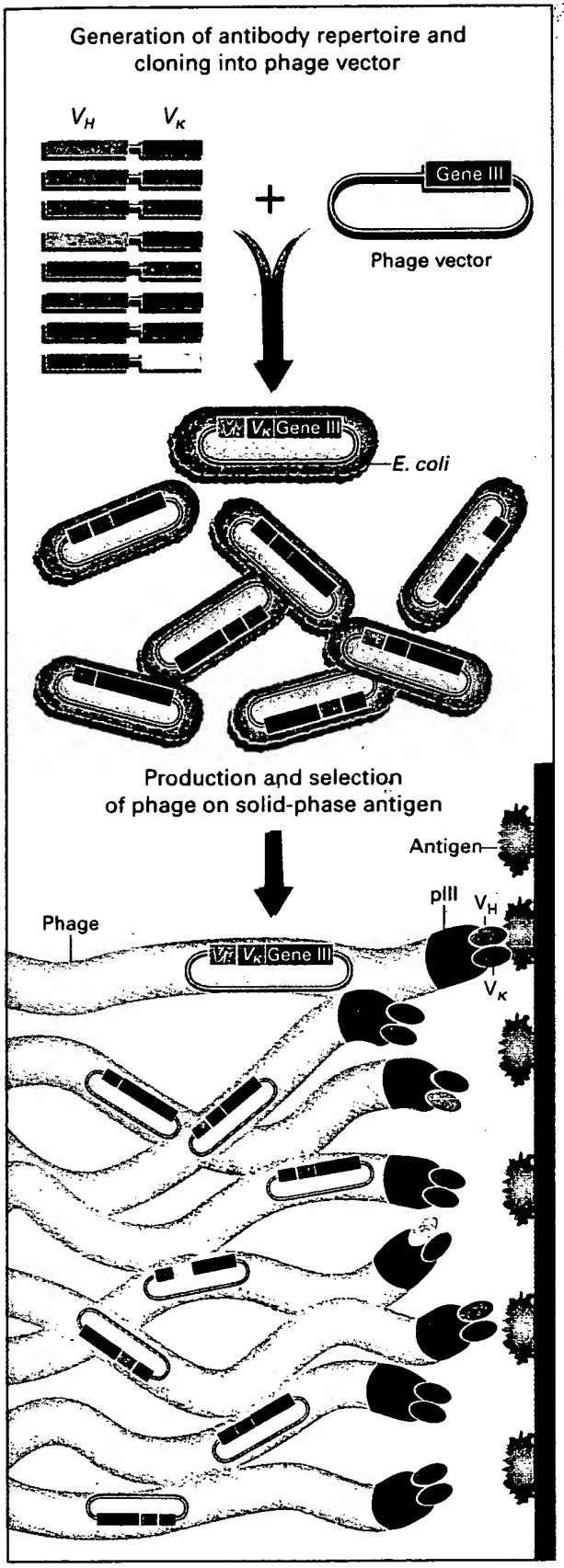


Figure 1. Generation of Antibodies in Vivo.

In the top panel, the random rearrangement of germ-line gene segments creates a repertoire of more than a million B cells with different immunoglobulin heavy-chain (V_H) and kappa light-chain (V_K) variable (*V*) genes. For the sake of simplicity, the lambda light-chain locus is not shown. The rearranged heavy-chain gene consists of 1 of approximately 50 V_H gene segments, 1 of 33 *D* gene segments, and 1 of 6 J_H gene segments. The random addition of nucleotides between the points at which the *V* and *D* segments and *D* and *J* segments meet greatly increases the diversity of the repertoire, which contains 10^6 to 10^9 different V_H genes. A similar rearrangement mechanism of V_K and J_K gene segments results in 10^3 to 10^4 different rearranged kappa light chains. The light chains are less diverse than the heavy chains, because there are no *D* segments in light-chain genes. The potential number of different antibodies created by gene rearrangements, which is the product of the numbers of diverse V_H and V_K segments, far exceeds the number of different B cells in the body. The rearranged genes are expressed as membrane-anchored immunoglobulins on the surface of the B cell, where they function as antigen receptors (middle panel). In the bottom panel, when antigen binds to the surface immunoglobulin, the B cell is stimulated to proliferate and differentiate into plasma cells.

The inset shows the IgG antibody fragments in detail. The modular structure of antibodies makes it possible to produce small antigen-binding fragments that can be expressed in *E. coli*. The Fab fragment consists of the V_H domain and the first domain of the constant region (C_H1) paired with V_K and the light-chain constant (C_K) domains. The single-chain Fv fragment consists of a single polypeptide chain with the V_H and V_K domains connected by a flexible peptide linker. The linker keeps the noncovalently linked variable domains from dissociating at physiologic concentrations.



phage antibodies in the mixture. The eluted phages are used to infect a fresh batch of *E. coli*, which produce more phages for the next round of selection. Repeated rounds of selection can isolate antigen-binding phages that were present at the start of the process at frequencies of less than 1 in a billion.

APPLICATIONS OF PHAGE ANTIBODIES

By exploiting strategies used by the immune system, phage libraries can produce antibodies with many clinically applicable immunochemical specificities. For therapeutic purposes, human antibodies can be produced without immunization by phage-antibody libraries that contain human *V* genes. From such libraries, many different antibodies can be isolated against virtually any antigen. Phage antibodies have a number of advantages over monoclonal antibodies produced from hybridomas. First, the amino acid sequences of the antibodies are entirely human, making them less immunogenic than murine antibodies or monoclonal antibodies that are mouse–human chimeras. Second, in vivo mechanisms that normally get rid of self-reactive antibodies are avoided, because selection occurs entirely in vitro. This makes it possible to produce human antibodies against human antigens or antigens conserved between species, thereby exposing therapeutic targets that have previously not been approachable with antibodies. Third, the immunoglobulin *V* genes are already cloned, and *E. coli* typically express the antibody fragments well. These features facilitate subsequent manipulation of the genes in ways that increase the affinity of the antibodies, change their fine specificity, and alter their size or valency. Furthermore, genetic engineering can be used to fuse the antibodies to cytolytic proteins that enhance the therapeutic effect of the antibodies. To increase the

Figure 2. Strategy for Antibody Production with Phage Libraries.

In the top panel, a repertoire of single-chain Fv genes (*scFv*) is generated with the polymerase chain reaction. The repertoires are constructed either from V_H and V_K genes that had been rearranged in vivo or from *V* gene segments that are rearranged in vitro (not shown). The repertoire of *scFv* genes is cloned into a phage vector in a way that fuses the *scFv* gene to a gene (gene III) that encodes a protein (pIII) expressed on the phage surface. In the middle panel, the vector directs *E. coli* to produce phage antibodies, which have on their surface a functional scFv fused to pIII. Inside each phage antibody is the vector DNA containing the gene for the scFv. Phage antibodies binding a specific antigen can be separated from nonbinding phage antibodies by affinity chromatography on immobilized antigen (bottom panel). A single round of selection increases the number of antigen-binding phage antibodies by a factor ranging from 20 to 1000, depending on the affinity of the antibody. Eluted phage antibodies are used to infect *E. coli*, which then produce more phage antibodies for the next round of selection. Repeated rounds of selection make it possible to isolate antigen-binding phage antibodies that were originally present at frequencies of less than 1 in a billion.

affinity of antibodies, the sequence of an immunoglobulin *V* gene in an antigen-binding phage antibody is mutated; phage antibodies that bind to the antigen with increased affinity can then be selected from the mutants produced. In this way, antibody affinity has been increased more than a thousandfold. Genetic manipulations make it easy to tailor antibodies to a specific application. For cancer therapy, for example, genetically engineered, small, high-affinity antibody fragments can target tumors more specifically than larger IgG antibodies.

Phage antibodies are also useful as diagnostic reagents and drug-discovery tools in the research laboratory. The ability to fine-tune specificity and affinity for diagnostic purposes will permit the identification of closely related molecules that occur in low concentrations in serum. In addition, the fusion of antibodies with detecting reagents, such as alkaline phosphatase, results in single-step in vitro diagnostic reagents. The ability to produce phage antibodies relatively quickly (within a period of two weeks) will lead to their widespread use as drug-discovery tools and research reagents, a particular advantage given the vast number of new genes being identified by genome-sequencing projects. Phage antibodies provide a rapid method for detecting the gene product and identifying its location. Moreover, the *scFv* gene can be subcloned, expressed intracellularly in eukaryotic cells, and targeted to subcellular compartments where the antibody can neutralize (knock out) the antigen to which it binds. This technique will help pinpoint the function of newly found genes. Phage libraries can produce antibodies against murine proteins, which is difficult to do with hybridoma-based technology. This feature of phage antibodies could allow the development of murine models of human diseases without the need for transgenic mice. Phage antibodies can also be used as probes to identify novel molecules directly, by selecting libraries on intact cells.

Although the technology is only five years old, the first generation of phage antibodies is already being used in phase 1 clinical trials. In the future, the only impediment to new clinical applications will be the investigator's imagination.

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